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I, Shiro TERASAKI
Japanese Patent Attorney
Registered No. 9265

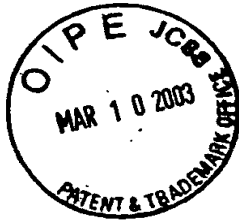
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Applicant(s): CHUGAI SEIYAKU KABUSHIKI KAISHA

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A METHOD FOR SCREENING PHARMACEUTICALS AND A METHOD FOR
MOLECULAR DESIGNING SAID PHARMACEUTICALS
(Number of Claims) 13
(Inventor)
(Residence or Address)
13-2, Motomachi, Tokorozawa-shi, Saitama, Japan
(Name) Shigeaki KATO

(Inventor)
(Residence or Address)
3-12-6, Sakuragaoka, Tama-shi, Tokyo, Japan
(Name) Jun YANAGISAWA

(Applicant)
(Identification Number) 000003311

(Name) CHUGAI SEIYAKU KABUSHIKI KAISHA

(Attorney)

(Identification Number) 100088155

(Patent Attorney)

(Name) Yoshiki HASEGAWA

(Attorney)

(Identification Number) 100089978

(Patent Attorney)

(Name) Tatsuya SHIODA

(Attorney)

(Identification Number) 100092657

(Patent Attorney)

(Name) Shiro TERASAKI

(Attorney)

(Identification Number) 100094318

(Patent Attorney)

(Name) Koichi YAMADA

(Attorney)

(Identification Number) 100089901

(Patent Attorney)

(Name) Kazuo YOSHII

(Attorney)

(Identification Number) 100107191

(Patent Attorney)

(Name) Noriaki NAGAHAMA

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[NAME OF DOCUMENT] Specification

[TITLE OF INVENTION] A METHOD FOR SCREENING PHARMACEUTICALS
AND A METHOD FOR MOLECULAR DESIGNING SAID PHARMACEUTICALS
[WHAT IS CLAIMED IS]

5 [Claim 1]

A method for screening pharmaceuticals comprising
detecting cross talk between intracellular signal from TGF-
 β family receptor and an intranuclear receptor.

 [Claim 2]

10 A method for screening pharmaceuticals which induce
cross talk between intracellular signal from TGF- β family
receptor and an intranuclear receptor.

 [Claim 3]

15 A method for screening pharmaceuticals which inhibit
cross talk between intracellular signal from TGF- β family
receptor and an intranuclear receptor.

 [Claim 4]

20 A method for screening pharmaceuticals comprising
detecting interaction between an intranuclear receptor and
Smad molecule.

 [Claim 5]

The method for screening pharmaceuticals as claimed
in Claim 4, characterized in that said interaction with said
Smad molecule is detected by using the Two-Hybrid System.

25 [Claim 6]

The method for screening pharmaceuticals as claimed

in Claim 4, characterized in that said interaction with said Smad molecule is detected by using antigen-antibody reaction.

[Claim 7]

5 A method for screening of pharmaceuticals comprising detecting interaction between a transcription coupling factor and Smad molecule.

[Claim 8]

10 The method for screening pharmaceuticals as claimed in Claim 7, characterized in that said transcription coupling factor is CBP and/or p300.

[Claim 9]

15 A method for screening of pharmaceuticals comprising detecting interaction among an intranuclear receptor, Smad molecule and an transcription coupling factor.

[Claim 10]

 The method for screening pharmaceuticals as described in Claim 9, characterized in that said transcription coupling factor is CBP and/or p300.

20 [Claim 11]

 The method for screening pharmaceuticals according to any one of claims 1 to 10, wherein the intranuclear receptor is vitamin D receptor.

[Claim 12]

25 The method for screening pharmaceuticals according to any one of claims 1 to 11, wherein Smad molecule is Smad3.

[Claim 13]

A method for molecular design for pharmaceuticals according to any one of claims 1 to 12 characterized by using steric structural data of the binding site of Smad molecule with an intranuclear receptor and CBP and/or p300 as a transcription coupling factor.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field to Which the Invention Belong]

This invention relates to a method for screening and molecular designing pharmaceuticals useful for various diseases on bone, cartilage, liver, kidney, skin, immune system, central system and the like, or benign tumor, malignant tumor, hyperplasia, fibrosis and the like.

[0002]

[Prior Art]

It has been attempted to develop TGF- β super family molecule itself, or antagonist or agonist thereof as a pharmaceutical based on focusing the function of TGF- β super family, and some pharmaceutical screening methods therefor have been proposed.

[0003]

For example, (1) as a method for screening by using cell system, there are screening methods for agonist or antagonist by using the activity of TGF- β super family in growing, differentiating, killing or maintaining animal

cells as an indicator. Those methods are, for example, (i) a screening method using inhibition activity of TGF- β on the growth of a tumor cell, or propagation accelerating activity of it on the growth of another tumor cell as an indicator, (ii) a screening method focusing on the immuno suppression activity of TGF- β , and (iii) a screening method focusing on production accelerating effect on extracellular matrix by TGF- β . Furthermore, (2) a screening method focusing on the interaction between TGF- β super family and TGF- β receptor super family is known.

[0004]

However, it is known that TGF- β super family has significantly wide-ranging functions, and thus, a mere agonist or antagonist for TGF- β super family may have a problem such that main function and side function can not be sufficiently differentiated, and consequently they are not sufficient for a screening method for pharmaceuticals. That is, the above screening method can not differentiate only main function from other functions, and very likely screen materials with low specificity not to be an candidate for pharmaceuticals. Furthermore, even if a useful candidate for pharmaceuticals could be screened, it still remains difficulty to find guideline for synthesizing a useful derivatives and to do molecular designing.

[0005]

[Problems to be Solved by the Invention]

An object of the present invention is to provide a novel method for screening pharmaceuticals, which overcomes the above prior art's problems.

[0006]

5 [Means for Solving the Problem]

As a result of intensive efforts to overcome the above-mentioned problems, the present inventor succeeded to develop a screening method for pharmaceuticals (inhibitor, accelerator etc.) with more specificity and a method for
10 molecular designing them, based on a novel principle by focusing on intracellular signal transfer mechanism of TGF- β receptor super family, and accomplished this invention.

[0007]

Although a pathway via Smad molecule and the MAP kinase
15 pathway via TAB1, TAK1 and the like have been known as an intracellular signal transfer mechanism of TGF- β receptor super family, it is little understood how the expression of the targeted gene is finally controlled by these pathways. Especially, it is known that Smad molecule affects
20 transcription control in nucleus, but a transcription control factor interacted with Smad molecule in higher animal cell has not been determined.

[0008]

The present inventor demonstrated that the
25 interaction among Smad molecule, intranuclear receptor and transcription coupling factor is important physiologically

in the transcription control mechanism of Smad molecule.
Under the above-mentioned knowledge, the present inventor
developed a method for screening and a method for molecular
designing pharmaceuticals based on the novel principle which
5 focuses on the transcription control mechanism via the
intracellular signal transfer mechanism of TGF- β receptor
super family.

[0009]

In the signal transfer pathway of Smad, as a
10 pharmacofunction being presumable from the prior art, there
are (i) phosphorylation into Smad2, 3 and the like (obtaining
transition ability into nucleus), (ii) dimer formation of
Smad molecule, and (iii) discovering of inhibitor against
the signal transfer pathway such as Smad 6, 7, or controlling
15 of function of the pathway. However, because these
pharmacofunctions are appeared in cytoplasm but not in nucleus
and the pathway is well conserved, a screening method for
pharmaceuticals based on such functions is not suitable to
search pharmaceuticals with high specificity. Furthermore,
20 other than the above (ii), the concrete interaction between
molecules has not been clarified, and thus a theoretical
approach for molecular designing based on the structure is
not appropriate.

[0010]

25 On the other hand, in connection with the
transcription control by Smad molecule, the present inventor

obtained the knowledge about the interaction of Smad molecule with intranuclear receptor or CBP/ p300, thereby clarified a tangible partner of Smad. According to such knowledge, the present inventor developed a screening method for pharmaceuticals with specified site of action and high specificity. By using the above method, it becomes possible to find useful pharmaceutical candidates and to provide a guideline or molecular designing for synthesizing more useful materials. Based on such knowledge, using the analysis method and numerical calculating modeling such as known techniques X-ray crystallographic structure analysis and NMR (nuclear magnetic resonance).

[0011]

Furthermore, base on the above principle, it becomes possible to develop a screening method for an agonist or an antagonist of intranuclear receptor (for example, vitamin D receptor) with higher specificity. Since intranuclear receptors are distributed over various tissues, it is impossible to avoid some side effect of agonist or antagonist type pharmaceuticals screened by the prior method, which such intranuclear receptors are simply targeted. However, upon focusing on the interaction between Smad molecule and intranuclear receptors, according to the present invention, it is possible to inhibit or accelerate only the specific transcription control using Smad as a transcription coupling factor. Therefore, it becomes possible to search excellent

pharmaceuticals with good efficiency and specificity
(targeting various disease of bone, skin, liver, kidney,
immune system, center system and the like, or benign tumor,
malignant tumor, hyperplasia or fibrolysis) in addition to
5 the known usefulness of intranuclear receptor agonist or
antagonist.

[0012]

In other words, the present invention provides a
method to develop a novel therapeutic agent based on the
10 novel knowledge of the interaction of Smad molecule with
intranuclear receptor or with CBP/p300.

[0013]

The present invention will be hereinafter explained
in detail along with some embodiment.

15 [0014]

[Embodiment]

A battery of factors belonging to TGF- β super family
such as TGF- β and BMP show various physiological functions ,
and they act as aggravation factor or amelioration factor
20 in various disease of bone, cartilage, liver, kidney, skin,
immune system and the like or carcinoma. On the other hand,
functions via vitamin D3, retinoic acid, steroid hormone
and receptors thereof (intranuclear receptor/steroid
hormone receptor super family) are also various, and target
25 tissues thereof are overlapped those of the above TGF-
 β super family.

[0015]

The present inventor found that the intracellular signal of TGF- β affects the transcription control of intranuclear receptors, that such function is expressed via intracellular signal transfer molecule of TGF- β , Smad, and that Smad3 affects particularly to transcription activity of VDR as transcription coupling factor and intermediates the interaction between VDR and CBP/p300.

[0016]

Furthermore, the present inventor found that Smad3 directly binds to CBP/p300 which is thought to be indispensable to activation of almost all intranuclear receptors, and thereby elucidated the cross-talk at molecular level between TGF- β intracellular signal and wide-ranging intranuclear receptor transcription control.

[0017]

Upon elucidation of the cross-talk between TGF- β intracellular signal and intranuclear receptor transcription control, an entirely different approach to create therapeutic agent involving TGF- β and intranuclear receptor, especially VDR becomes possible.

[0018]

In other words, an pharmaceutical agent affecting intracellular signal of TGF- β and intranuclear receptor transcription activity improves tissue specificity and efficiency, which have not been accomplished heretofore. And the

pharmaceutical agent enables to establish various kind of screening system based on molecular interaction. Thus, materials found by the screening method based on such knowledge include effective therapeutic agents for diseases of bone, cartilage, liver, kidney, skin, immune system and central system, or benign tumor or malignant tumor, hyperplasia, fibrosis and the like.

[0019]

While TGF- β employs Smad2 or 3 and Smad4 for intracellular signal transfer, BMP belonging to TGF- β super family employs Smad1 and Smad4 having similar structure for intracellular signal transfer. The present invention provides a novel screening method using mainly TGF- β , Smad3, CBP/p300 and various intracellular receptors, but it can be easily assumed to be common to BMP and Smad1.

[0020]

Because Smad3 binds to CBP/p300 directly, this invention is also applicable to screening of transcription activity control materials of transcription activating factors (CREBP, AP-1 and the like) other than intranuclear receptors.

[0021]

The screening method of this invention will be exemplified.

[0022]

Screening method (I):

Based on the knowledge found by the present inventor, it

is possible to screen cross talk between intracellular signal from TGF- β family receptors and intranuclear receptors and to screen (search) materials affecting the cross talk (see Example 1).

5 [0023]

Screening method (II):

A screening method is possible by using the Two-Hybrid System. In order to screen materials affecting the interaction (binding) between intranuclear receptor and Smad molecule, the Two-Hybrid System (Gyuris, J., Cell, 1993, 75: 791-803; Golemis, E. A., Current Protocols in Molecular Biology [John Wiley & Sons, Inc.], 1996, Ch. 20.0 and 20.1) can be used.

[0024]

15 As a transcription activating domain used for the Two-Hybrid System, for example, GAL4 transcription activating domain (Brent, R., Cell, 1985, 43: 729-736), [Bicoid], [c-Fos], [c-myc], [v-Myc], [B6], [B7], [B42] (Golemis, A. E., Mol. Cell Biol., 1992, 12: 3006-3014) or [VP16] (CLONTECH Corp., Mammalian MATCHMAKER Two-Hybrid Assay Kit) may be listed.

[0025]

A DNA binding domain used for the Two-Hybrid System, for example is identified in transcription factors such as GAL4 (Giniger, E., Cell, 1985, 40: 767-774), p53 (Chumakov, P. M., Genetika, 1988, 24: 602-612), GCN4 (Hinnenbush, A. G.,

Proc. Natl. Acad. Sci., 1984, 81: 6442-6446), VP16
(Triezenberg, S. J., Genes. Dev., 1988, 2: 718-729)、ReloA
(Nolan, G. P., Cell, 1991, 64: 961-969)、Oct-1 (Strum, R.
A., Genes. Dev., 1988, 2: 1582-1599)、c-Myc (Watt, R., Nature,
5 1983, 303: 725-728)、c-Jun (Angel, P., Cell, 1988, 55:
875-885)、MyoD (Write, W. E., Cell, 1989, 56: 607-617).

[0026]

The principle to screen materials affecting the
binding between proteins A and B by using the Two-Hybrid
10 System is explained hereinbelow. An expression vector for
the fusion protein of the above-mentioned transcription
activating domain and the protein A, an expression vector
for the fusion protein of the above-mentioned DNA binding
domain and the protein B, and an eucaryotic host having an
15 expression unit comprising an appropriate reporter gene in
its nucleus are prepared. The reporter gene is constructed
as an expression unit consisting of promoter region which
is activated when both fusion proteins bind together and
the reporter gene linked to the promoter region at the
20 downstream of the region.

[0027]

It is possible to screen materials affecting the
binding of proteins A and B by detecting the expression of
the reporter gene and selecting a material affecting the
25 amount of the expression.

[0028]

An example of the expression vector may be used any expression vector without any restriction as long as it can stably express proteins coded by (1) fusion DNA fused with DNA coding the above-mentioned transcription activating domain and an intranuclear receptors or Smad, and (2) fusion DNA fused with DNA coding the above-mentioned DNA binding domain and an intranuclear receptor or Smad in eucaryote hosts. An example of intranuclear receptor is preferably VDR, but other intranuclear receptor/hormone receptors, such as $RAR\alpha$, β , γ , $RXR\alpha$, β , γ , $PPAR\alpha$, β , γ , ER, AR, PgR, GR and the like may be used without any restriction. Any DNA coding an intranuclear receptor may be used as long as it includes DNA coding ligand / hormone binding domain. An example of Smad molecule is preferably Smad3, but DNA coding Smad2 or other molecules belonging to Smad family may be used without any restriction.

[0029]

Any expression vector for reporter gene may be used as long as it includes the promoter region activated upon the binding of the both fusion proteins and reporter gene linked to the promoter region at the downstream of the gene and can express the reporter gene products specifically and efficiently when the both fusion proteins bind together (see Example 2).

[0030]

Screening method (III):

A screening method is possible by employing antigen

antibody reaction. After Smad molecules and intranuclear receptors are expressed in cells and those cells are cultivated with a material to be tested for a certain period, cells are crushed to prepare cell lysate. Effect of the material to be tested against the interaction (binding) of both molecules (Smad-intranuclear receptor) can be detected by immune precipitation with antibody against one of those molecules, and detecting or quantifying another molecule involved in the immune precipitate with a immunological technique. The screening for materials inducing the binding of the both molecules is easily accomplished by adding a material to be tested alone to the above-mentioned culture system and performing the above-mentioned experiments, and then comparing to the immune precipitate obtained from cells without the material. Material modifying (accelerating or suppressing) or inhibiting the binding of the both molecules can be screened by adding an appropriate agonist/hormone of an intranuclear receptor and a material to be tested to the above-mentioned culture system simultaneously and performing the above described experiments, and then by comparing to immune precipitate obtained from cells without the material.

[0031]

Smad and intranuclear receptors may be used by expressing as a fusion protein fused with tag such as FLAG-, HA-, His-, Fc portion of immunoglobulin, GST-, GFP or labeled

protein/ peptide. In such case, an antibody used for immune precipitate or immunological detection may be an antibody recognizing those tags. Instead of immune precipitation with an antibody, the targeted protein complex can be captured by using Ni or glutathione immobilized on a solid surface such as beads. Furthermore, in order to detect the targeted protein, by using the feature of the fusion tag/labeled protein/peptide, it is possible to detect the targeted protein based on its enzyme activity and fluorescence activity. As the intranuclear receptor, a ligand binding domain alone, or any fusion protein including a ligand binding domain can be used.

[0032]

When the targeted protein in the Smad - intranuclear receptor complex obtained by immune precipitate or the above-mentioned method is to be detected or quantified, separation by SDS-polyacrylamide gel electrophoresis and detection by a western blotting can be used. ELISA or RIA can be also used to quantify it.

[0033]

As a simple laboratory procedure, it is possible to immobilize an antibody which recognizes one of the fusion protein molecules or the fusion protein itself, Ni, glutathione and the like on ELISA plate or beads, to capture Smad - intranuclear receptor complex from the above-mentioned cell lysate, and to detect or quantify the targeted protein molecule in the captured complex

by using an antibody recognizing another protein molecule. For example, like commonly used in detection procedures, a direct assay using an antibody labeled with an enzyme or radioisotope, an indirect assay using labeled secondary antibody, or an
5 biotin-avidin assay which uses biotin-labeled antibody and quantifies with labeled avidin can be used. Where one of the protein molecules is expressed as a fusion protein with fluorescence protein such as GFP, the interaction (binding condition) of Smad and intranuclear receptor can be determined by measuring the
10 fluorescence directly after capturing Smad - intranuclear complex on solid surface with for example an antibody recognizing another protein molecule (see Example 3).

[0034]

Screening Method (IV):

15 Based on the interaction of Smad and intranuclear receptors, materials affecting the interaction can be screened in cell-free system (see Example 4).

[0035]

Screening method (V):

20 A screening can be done by detecting the interaction of transcription coupling factor and Smad molecule. CBP and P300 act as a transcription coupling factor mediating transcription activity from many transcription control factors including intranuclear receptors. VDR does not bind
25 CBP/P300 directly. However it is demonstrated that VDR binds CBP/P300 via Smad3 as described below.

[0036]

Consequently, it is necessary to sufficiently consider functions over the binding of Smad molecule and CBP/P300 in order to screen materials affecting the transcription control by Smad molecule and intranuclear receptor. Thus, a screening system for materials affecting the binding Smad molecule and CBP/p300 can be established by applying the following examples.

[0037]

The experimental result using dominant negative Smad3 suggests that Smad3 accelerates transcription control by cAMP, phorbol ester or PDGF (Istvan Mucsi, et al., BBRC; 232, 517-521, 1997). However, since the mechanism has not been demonstrated, it has been difficult to screen materials modifying such wide-ranging functions of Smad3. The present invention makes such wide-ranging functions via Smad3 understandable systematically as the interaction of Smad molecule and CBP/p300. Consequently, it becomes possible to develop a screening system for materials affecting the binding of the above-mentioned both molecules.

[0038]

The method uses GST-pull down method (described supra) employing GST-Smad fusion molecule and labeled CBP/p300, and can detect materials modifying the binding of the both molecules by adding a material to the reaction system upon reaction of the both molecules. Also, GST-pull down method employing GST-CBP and

GST-p300 fusion protein and labeled Smad molecule can be used.

[0039]

Any system which can measure the interaction
(binding) between proteins other than GST-pull down method
described above enables to screen materials accelerating
or suppressing the interaction of Smad molecule and CBP/p300.
Such system includes both cell and cell-free system, and
can use easily ELISA, RIA, SPA method, BIAcore™,
fluorescence polarization method, and the Two-Hybrid System.
A partial peptide of Smad molecule or CBP/p300 molecule can
be used. In addition, Smad, CBP/p300 molecule or partial
peptide of them can be used as a fusion protein fused with
tag such as FLAG-, HA-, His-, Fc portion of immunoglobulin,
GST-, GFP or labeled protein/peptide.

[0040]

[Examples]

This invention will be hereinbelow explained in
detail by some examples. However, this invention is not
limited by the following examples.

[0041]

(Example 1)

TbetaR-I TD in which a gene coding constructively
active modified TGF- β type I receptor (T β RI-TD) was
introduce into higher animal expression vector was used to
introduce TGF- β signal into cells constructively.

[0042]

The gene coding androgen receptor (AR), the gene coding vitamin D₃ receptor (VDR), or the gene coding retinoic acid receptor (RAR) were introduced into higher animal expression vector pSG5, respectively, and each expression plasmids for respective receptors was constructed (pSG5-AR, pSG5-VD or pSG5-RAR), and those plasmids were used in the following experiments. Plasmid with only each expression vector was used as control.

[0043]

Reporter gene expression plasmid was used in which the sequence corresponding to each hormone receptor/ intranuclear receptor was inserted into CAT reporter gene expression vector.

[0044]

COS-1 cells which were cultured in Dulbecco's modified Eagle's medium- 5 % dextran-coated activated charcoal treated fetal calf serum without phenol red were used for the following experiments. COS-1 cells were cultivated in a 10 cm diameter Petri dish. At the point that cells saturation density was reached to 40- 50 %, the cells were combined with above-mentioned plasmids according to each purpose to make 20 μ g in total, and the plasmids were introduced into cells by calcium coprecipitation method. As a internal reference, 3 μ g of the expression vector pCH110 (Pharmacia) of β -galactosidase gene was introduced into cells simultaneously, and the introduction efficiency among

respective Petri dishes was adjusted based on the amount of expression of the gene. Each material to be tested such as testosterone, $1,25(\text{OH})_2\text{VD}_3$ and retinoic acid was added into Petri dish one hour after the gene introduction, and thereafter was also added upon medium exchange.

[0045]

Twenty-four hours after the gene introduction, cells were washed with fresh culture medium and continued to be cultured for additional 24 hours. Cells were crushed with freeze and thaw method to prepare cell extract. CAT activity was measured and concurrently β -galactosidase activity were measured to adjust the data.

[0046]

The result showed that the transcription activity made by the addition of testosterone or retinoic acid via AR or RAR was slightly reinforced by signal from TGF- β . The transcription activity made by the addition of $1,25(\text{OH})_2\text{VD}_3$ via VDR was significantly reinforced by signal from TGF- β (Fig. 1). These results demonstrate that intracellular signal of TGF- β affects (reinforces) transcription activity of hormone receptor/ intranuclear receptor.

[0047]

Accordingly, by using this analysis system, it is possible to screen materials affecting transcription control mechanism of hormone receptor/ intranuclear

receptor mediated by intracellular signal of TGF- β .

[0048]

In other words, it is possible to screen hormone agonist or antagonist which have different dependency on TGF- β signal - intranuclear receptor interaction by the method which comprises addition of any various material to be tested into the system instead of physiological ligands such as testosterone, 1,25(OH)₂VD₃ or retinoic acid, or together with them in the presence or absence of signal of TGF- β , and comparison the transcription activity of each receptor. It is likewise possible to screen accelerator or inhibitor of TGF- β signal - intranuclear interaction.

[0049]

(Example 2)

Two-hybrid analysis using higher animal cells

COS-1 cells which were cultured in Dulbecco's modified Eagle's medium - 5 % dextran-coated activated charcoal treated fetal calf serum without phenol red were used for the following experiments. COS-1 cells were cultivated in a 10 cm diameter Petri dish. At the point that cell saturation density was reached to 40 - 50 %, 20 μ g of the following plasmid in total was introduced into cells by calcium phosphate coprecipitation method. As an internal reference, 3 μ g of the expression vector pCH110 (Pharmacia) of β -galactosidase gene was introduced simultaneously, and introduction efficiency among respective Petri dishes was

adjusted based on the amount of the expression of the gene. Each material to be tested such as VD₃ was added into Petri dish one hour after the gene introduction, and thereafter was also added upon medium exchange. Twenty-four hours after the gene introduction, cells were washed with fresh culture medium and continued to be cultured for additional 24 hours. Cells were crushed with freeze and thaw method to prepare cell extract. CAT activity was measured and β -galactosidase activity was measured concurrently to adjust the resulting data.

[0050]

As a plasmid for the two-hybrid analysis, the gene coding D, E, F domains of VDR was introduced into pM (Clontech) vector and thereby the expression plasmid of GAL4 DNA-binding domain - VDR (DEF) fusion protein was constructed. Also, genes coding Smad2 and 3 were introduced into pVP16 (Clontech) vector and those were used as expression plasmid for VP16 - Smad2 or VP16 - Smad3 fusion proteins. As a reporter gene expression plasmid, 17M5-CAT in which CAT gene was inserted into 17M5 (including 5 times of UAS 17mer of GAL-4 recognition site). These expression plasmids were introduced into COS-1 cells as described in above.

[0051]

In cells which expressed GAL4 DNA-binding domain - VDR (DEF) and VP-16-Smad2 or GAL4 DNA-binding domain - VDR (DEF) and VP-16-Smad3, stronger CAT activity increase

was detected in the presence of 1 nM of $1\alpha, 25(\text{OH})_2\text{VD}_3$, compared with that in the absence of $1\alpha, 25(\text{OH})_2\text{VD}_3$ (see Fig. 2). It was shown that Smad3 and VDR bonds each other in the presence of $1\alpha, 25(\text{OH})_2\text{VD}_3$.

5 [0052]

By using this analysis system, it is possible to screen materials reinforcing the interaction of Smad3 and VDR. It is also possible to screen materials inhibiting the binding of Smad2 or Smad3 and VDR recognized in the presence of VD_3 agonist such as $1\alpha, 25(\text{OH})_2\text{VD}_3$.

10

[0053]

(Example 3)

Immune precipitate method — immunoblotting method

15

The following two kinds of expression plasmids were introduced into COS-1 cells by the same procedure as set forth Example 1. One hour after the gene introduction, $1,25(\text{OH})_2\text{VD}_3$ was added into Petri dish to be 1 nM at its concentration or not added, and thereafter was also added at same concentration upon medium exchange. Twenty-four hours after the gene introduction, cells were washed with fresh culture medium and continued to be cultured for additional 24 hours. (1) pSG5-VDR in which VDR gene is inserted into higher animal expression vector pSG5, and (2) the plasmid in which FLAG-tagged Smad3 gene is inserted into higher animal expression vector pcDNA3 (Invitrogen) were used. After lysing cells, immune precipitation was done with

20

25

anti-FLAG monoclonal antibody M2 (IBI; Eastman Kodak).
Then, coprecipitated proteins were separated by 8%
SDS-polyacrylamide gel electrophoresis. Separated proteins
were transferred onto PVDF membrane (Bio-Rad), and then VDR
5 was detected by using rabbit anti VDR antibody and alkaline
phosphatase labeled anti rabbit IgG antibody (Promega).

[0054]

The results show that VDR was barely detected in the
example using cells without $1,25(\text{OH})_2\text{VD}_3$. On the other hand,
10 the VDR band was clearly detected in the example using cells
added with $1,25(\text{OH})_2\text{VD}_3$ (Fig. 3).

[0055]

By using this analysis system, it is possible to screen
materials accelerating the interaction (binding) of Smad3
15 and VDR. Furthermore, it is possible to screen materials
inhibiting the interaction (binding) of Smad3 and VDR in
the presence of agonist such as $1,25(\text{OH})_2\text{VD}_3$ by adding any
materials into this culture system.

[0056]

20 (Example 4)

Detection system for interaction of VDR and Smad molecule
-GST-pull down analysis-

GST fused Smad3 or VDR (full length peptide 1-424,
partial peptides: amino acid sequence 115-424, 115-357,
25 115-300 and others, see Fig. 4) fused proteins (GST-Smad3
or GST-VDR) were expressed by using E.coli, and were captured

on beads with glutathione-Sepharose beads (Pharmacia) to accomplish affinity purification. On the other hand, ³⁵S-methionine-labeled Smad3 and VDR were prepared in cell-free system by using reticulocyte lysate translation system (Promega). GST-Smad3 or GST-VDR fixed on beads described above was incubated with ³⁵S-methionine-labeled VDR or Smad3. By using polyacrylamide gel electrophoresis, ³⁵S-methionine labeled VDR or Smad3 were analyzed.

[0057]

The results show that VDR partial peptides, 115-357 and 115-300 bound to Smad3. This binding did not require 1,25(OH)₂VD₃. On the other hand, full length VDR slightly bound to Smad3 in the presence of 1 nM of 1,25(OH)₂VD₃ (Fig. 5).

[0058]

The disjunction from the result of 1,25(OH)₂VD₃ in cell system is presumed that in cell system, in the presence of 1,25(OH)₂VD₃, intranuclear receptor transcription coupling factor (SRC-1/TIFII family protein) interacts with AF-2 core domain located near to C-terminal of VDR to result in the structure change of VDR and to make partial peptide of main central portion of VDR E domain (amino acid: 115-357, 115-300) possible to interact with (bind to) Smad molecule. In cell-free system, the strong binding of full length VDR and Smad molecule is not detected even though 1,25(OH)₂VD₃ is added into the reaction system because of lacking

transcription coupling factor.

[0059]

Because VDR(115-300) and VDR(115-357) are VDR partial peptides containing binding site with Smad3, it is possible to screen materials accelerating or inhibiting VDR -Smad interaction (binding) by adding a material to be tested into GST-pull down analysis system using GST-VDR(115-300) or GST-VDR(115-357) and labeled Smad3.

[0060]

Any analysis system other than GST-pull down method, in which interaction (binding) between proteins can be measured enables to screen materials accelerating or inhibiting the interaction of Smad molecule and intranuclear molecule. Such measurement systems are possibly ELISA, RIA, SPA method, BIACORE™, fluorescence polarization method. Furthermore, respective partial peptides of Smad molecule and intranuclear receptor molecule can be used.

[0061]

Moreover, respective partial peptides of Smad or intranuclear receptor molecule can be used as a fusion protein fused with tag such as FLAG-, HA-, His-, Fc portion of immunoglobulin, GST-, GFP or labeled protein/peptide.

[0062]

(Example 5)

Detection for interaction of Smad molecule and CBP/p300 -GST-pull down method-

GST fused Smad3 fusion protein (GST-Smad3) was expressed by using E.coli, and was captured on beads with glutathione -Sepharose bead (Pharmacia) to accomplish affinity purification. On the other hand,

5 ³⁵S-methionine-labeled CBP and p300 were prepared in cell-free system by using reticulocyte lysate translation system (Promega). GST-Smad3 fixed on beads described above was incubated with ³⁵S-methionine labeled CBP or p300. After accomplishing SDS-polyacrylamide gel electrophoresis,
10 ³⁵S-methionine labeled CBP or p300 was analyzed by using autoradiography. The results show that CBP and p300 bound to Smad3 directly (Fig. 6A and 6B).

[0063]

In this analysis system, when GST-Smad3 and
15 ³⁵S-methionine labeled CBP (GST-Smad3 and ³⁵S-methionine labeled p300) are reacted together, the resultant autoradiographical band of CBP or p300 would be increased or decreased in the presence of any material accelerating or inhibiting the binding of these molecules. By using such
20 change as indicator, it is possible to screen materials modifying (accelerating or inhibiting) the interaction of Smad3 and CBP/p300.

[0064]

[Effect of the Invention]

25 Based on the novel knowledge which intracellular signal transfer mechanism from TGF- β super family by TGF-

β super family stimulation do cross talk with intranuclear receptor or transcription control factor CBP/p300 via Smad molecule, a screening method for pharmaceuticals (such as inhibitor, accelerator) with high specificity can be accomplished.

[BRIEF DESCRIPTION OF DRAWINGS]

[Fig. 1]

Fig. 1 is a diagram showing the results of Example 1.

[Fig. 2]

Fig. 2 is a diagram showing the results of Example 2.

[Fig. 3]

Fig. 3 is electrophoresis patterns showing the results of Example 3.

[Fig. 4]

Fig. 4 is a diagram showing the results of Example 4.

[Fig. 5]

Fig. 5 is a diagram showing the results of Example

[Fig. 6]

Fig. 6A is electrophoresis patterns showing the results of Example 3 in the case of p300.

Fig. 6B is electrophoresis patterns showing the results of Example 3 in the case of CBP.

[NAME OF DOCUMENT] Abstract

[ABSTRACT]

[Object] To provide high specific methods for screening pharmaceuticals and for molecular designing.

5 [Means for Solving the Problem] Under the knowledge which two kinds of intracellular signal transfer mechanism of TGF- β super family receptor exist: a pathway via Smad molecule and a pathway via novel MAP kinase such as TAB1 and TAK1, and the knowledge of interaction of Smad molecule with
10 intranuclear receptor or CBP/p300 on the transcription control by Smad molecule, a screening method and a molecular designing method for pharmaceuticals with identified site of action and higher specificity can be accomplished.

[Representative Drawing] Fig. 1

Name of Documents

【書類名】 図面 Drawing

Fig. 1
【図号】

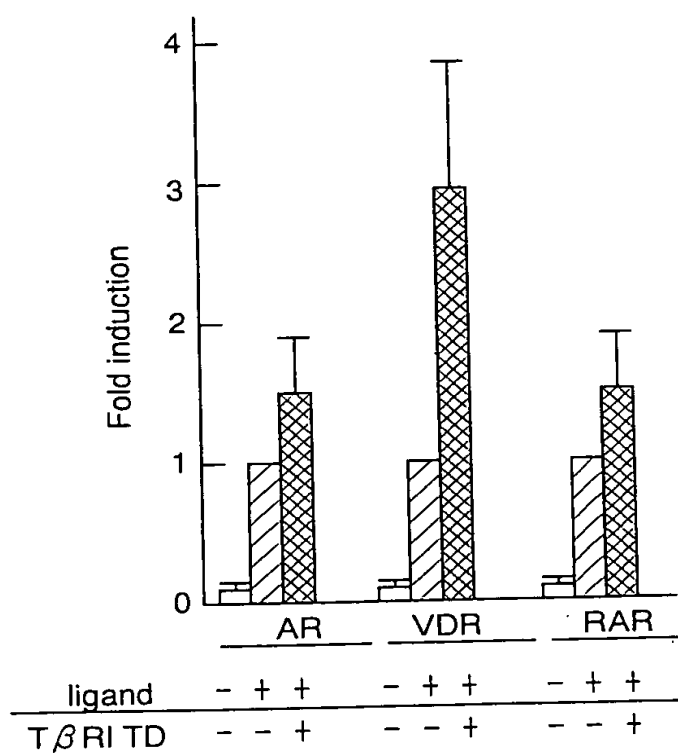


Fig. 2
【図2】

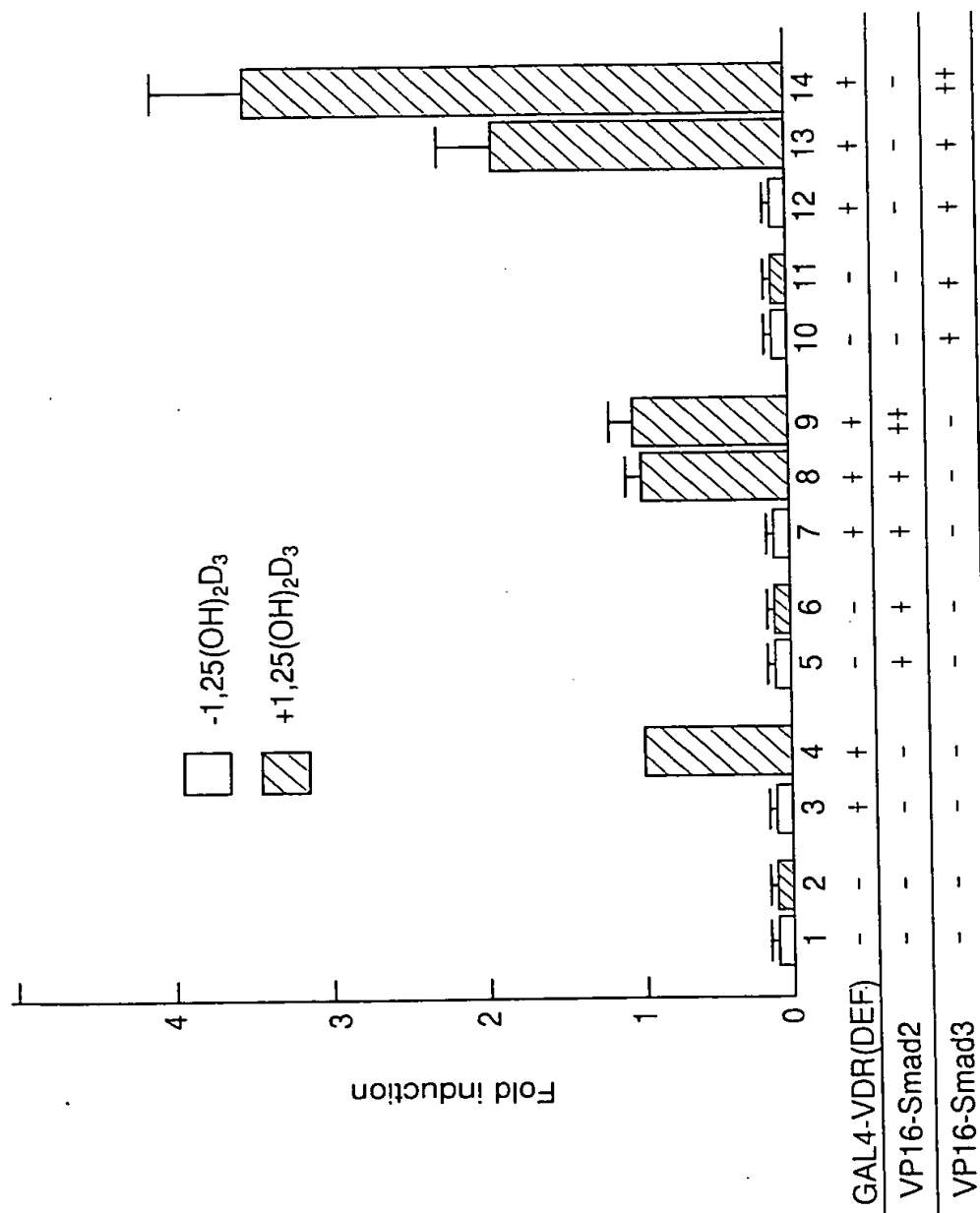


Fig. 3
【図3】

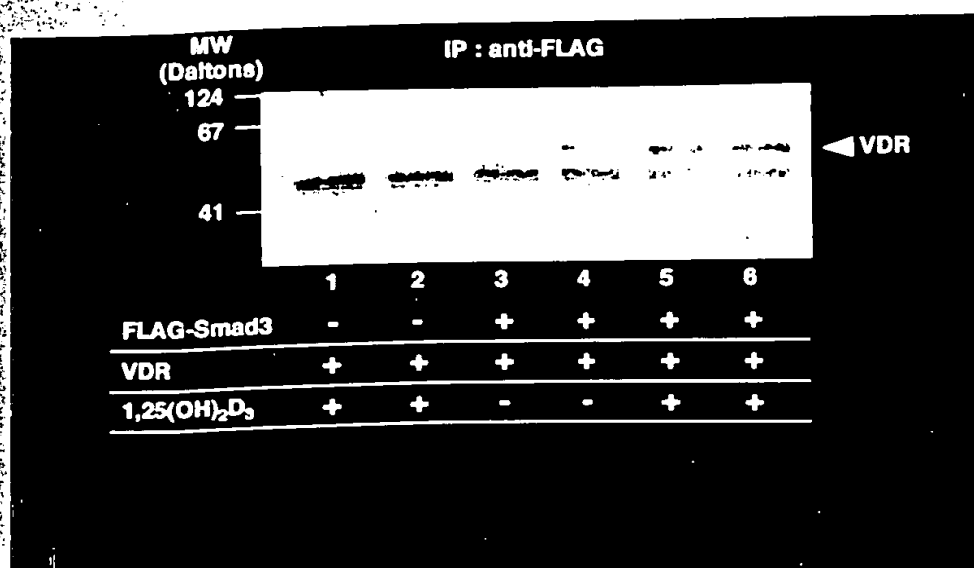


Fig. 4
【図4】








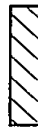







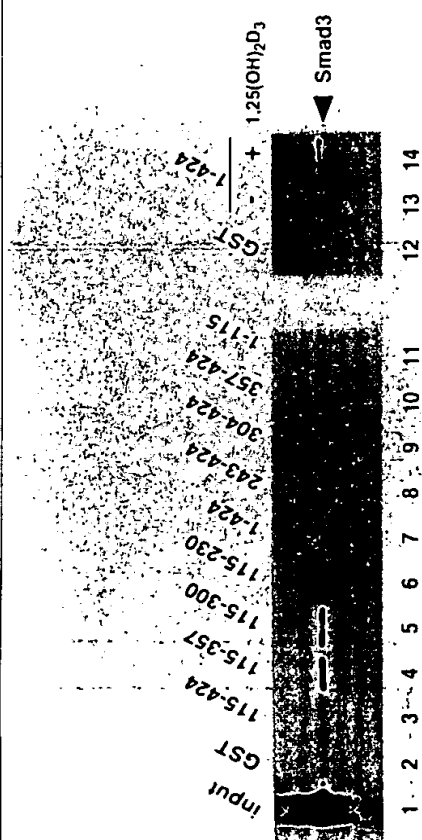
Construct				1,25(OH) ₂ D ₃	
A/B	C	D	E	F	
					-
					+/-
					+
					+
					-
					+/-
					+
					+
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					+/-
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Fig. 5
【図5】



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(6)

Fig. 6
【図6】

